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REVIEW ARTICLE

Flow cytometric analysis and sorting of plant chromosomes

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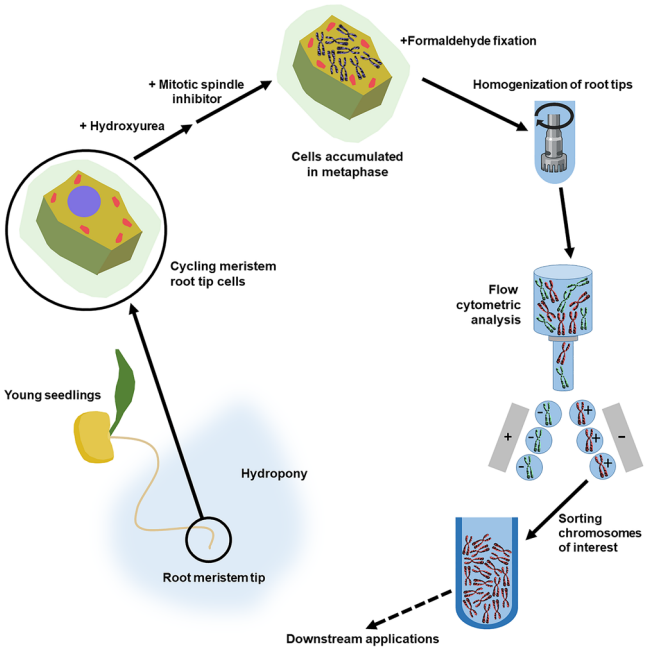
Abstract

Flow cytogenetics is a high-throughput technique that classifies large populations of mitotic chromosomes according to their fluorescence and light scattering as they move in a single file rapidly in a narrow stream of liquid. Flow karyotyping can detect structural and numerical chromosome changes, and chromosomes purified by sorting have been used to discover the three-dimensional organization of DNA, characterize the proteome, and describe the organization of the perichromosomal layer at the nanometre level. However, the most frequent and important use of flow cytogenetics has been the isolation of different chromosomes. Chromosome sorting has made it possible to dissect nuclear genomes into small and defined parts, therefore allowing targeted, simplified, and more economical genomic analyses. The uses of flow-sorted chromosomes for genome complexity reduction and targeted analysis have expanded hand-in-hand with the progress of molecular biology and genomics techniques. These uses include the targeted development of DNA markers, the construction of chromosome-specific DNA libraries, physical mapping, draft genome sequencing, and gene cloning.

Graphical abstract



Keywords Flow cytometry · Chromosome sorting · Genome sequencing · Gene cloning · Physical mapping

Jaroslav Doležel and Petr Urbiš contributed equally to this work.

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Introduction

In almost all eukaryotes, nuclear genome is distributed into two or more chromosomes [13]. This highlights the role of chromosomes in transferring genetic information to daughter cells and progenies. Splitting a genome to more chromosomes permitted its evolutionary expansion so that the length of individual chromosomes was kept under the biological limit [93]. In sexually propagated species, chromosomes allow generation of genetic variation by recombination and independent assortment in meiosis. Early studies on genome organization by the end of the 19th century concentrated on condensed chromosomes during mitosis and meiosis. As chromosomes may differ in size and structure, they constituted an important character of a species. Importantly, these studies led to the formulation of chromosome theory of heredity [103].

Since the first experiments, the main approach to study chromosomes has been the observation of chromosomes immobilized on a solid support using optical microscopy. This has been a fruitful path as the chromosomes could be observed at appropriate magnification and for as long time as needed, provided the method of staining, and/or labelling allowed it. Chromosome length and structure may be studied in detail, and images may be captured using a camera and processed digitally [1]. This way of studying chromosomes is necessarily laborious, time-consuming and requires trained staff, in particular when small alterations of chromosome length and structure are studied, such as those originating from translocations.

Despite these requirements, until today the optical microscopy remains the main tool to study genomes at chromosome level. However, it has been complemented by a variety of experimental approaches, including electron microscopy [118] and, more recently genomics tools such as hybridization on DNA arrays [52] and next generation sequencing [38]. This paper reviews the development and application of flow cytometry as another complementary approach to study chromosomes in plants, providing a unique link between cytogenetics, molecular biology and genomics.

Chromosomes in motion

The use of flow cytometry to analyze chromosomes instead of microscopy may seem contra intuitive as until recently, the method did not capture images of particles in flow. In fact, and as given below, the areas of the major use and impact differ from those of microscopy. This is because flow cytometry has been developed to analyze electric and/or optical properties of large populations of particles moving in a narrow stream of liquid, rather than capturing images.

Basic principles

As per the Coulter principle, when the electric conductivity of particles is quantified [26], is not relevant to chromosome analysis, we focus here on the analysis of optical properties, when light pulses are generated by the interaction of a light beam with chromosomes moving rapidly in a single file (Fig. 1). The parameters measured comprise light scattering and fluorescence. Light scattering is quantified either in the direction of the light beam, when the amount of scattered light is related to particle size, or in the right angle to the light beam, when the amount of scattered light is related to the internal structure (i.e. granularity) of a particle [65]. While there are two basic types of light scattering, forward and side, pulses of fluorescence, either intrinsic or extrinsic, may be measured simultaneously in different parts of optical spectrum and used to quantify molecular components in individual particles.

The ability to analyze large populations of particles at high speed (10^2–10^4/s) makes flow cytometry suitable for assessing heterogeneity and identifying minor subpopulations. These may be detected based on a single parameter, or a combination of multiple optical parameters. In addition to the quantification of multiple optical parameters, flow cytometry enables isolation (sorting) of subpopulations of interest. It is important that this is done at high speed during the analysis. Several technical solutions were designed to achieve separation of desired particles. These may be classified into two basic types: fluidic switch type, when the direction of the liquid stream is temporarily changed to collect particle(s) of interest, and droplet sorter, when the liquid stream is broken into droplets and droplets containing particles of interest are electrically charged and directed to a collection vessel by a passage through electrostatic field (Fig. 1). Only droplet sorters are suitable for chromosome sorting as small droplets (typically 1 nl volume) are sorted and the collected fraction is not excessively diluted as in case of the fluidic switch system.

Developing flow cytogenetics for plants

The principle of flow cytometry, when particles are characterized individually while moving in a liquid stream, is dictated by the character of samples, which must be suspensions of single particles. However, preparation of suspensions of intact mitotic chromosomes is a challenge. First, only a fraction of cells in animal and plant somatic tissues is dividing and have condensed chromosomes in cytoplasm. This is a problem as flow cytometry requires samples of a small

volume (ideally several hundred microliters) and the samples must be concentrated (containing 10^5–10^6 chromosomes per milliliter). Thus, cell populations from which samples are prepared must be enriched for cells at mitotic metaphase. Secondly, the chromosomes must be released from intact cells into an isolation solution. However, plant cells have a rigid cell wall which hampers chromosome release. This is generally not a problem in animals and human and it is thus not surprising that the first ever chromosome analyses using flow cytometry were done in Chinese hamster and human [27, 28, 101].

It took almost ten years after these pioneering reports before the first successful attempt to analyze plant chromosomes by flow cytometry [51]. As discussed by Doležel et al. [20] the delay was due to difficulties in achieving sufficient metaphase synchrony and in releasing chromosomes from cells with rigid walls. In their pioneering work, De Laat and Blaas [51] isolated chromosomes from suspension cultured cells of *Haplopappus gracilis*, and the same type of biological system was used to prepare chromosome suspensions from *Lycopersicon esculentum* and *L. pennellii* by Arumuganathan et al. [5] as well as from *Triticum aestivum* by Wang et al. [116]. In these studies, chromosomes were released by lysing synchronized cells in hypotonic buffer after enzymatic removal of their walls [5, 51, 116]. A range of difficulties prevented in vitro cell cultures from becoming a widely used, which includes cell type heterogeneity, karyological instability and difficulties to achieve high degree of cell cycle synchrony [5, 57, 94].

An alternative idea was to use protoplasts prepared from leaf mesophyll cells [11, 12]. As the cells are arrested in G_1 phase of cell cycle, it was expected that transferring protoplasts to a nutrient medium would induce synchronous transition through the cell cycle. However, after treating cultured protoplasts of *Petunia hybrida* with colchicine, metaphase indices of only about 10% cells were reached [11]. In this work, chromosomes were released by mechanically disrupting cells in the nutrient medium. Due to low mitotic synchrony and a need to optimize protoplast isolation protocol for particular species, this approach was not used in other species.

To avoid the difficulties associated with tissue cultures, Doležel et al. [16] developed a protocol for chromosome isolation from meristem root tips of young seedlings (Fig. 2).

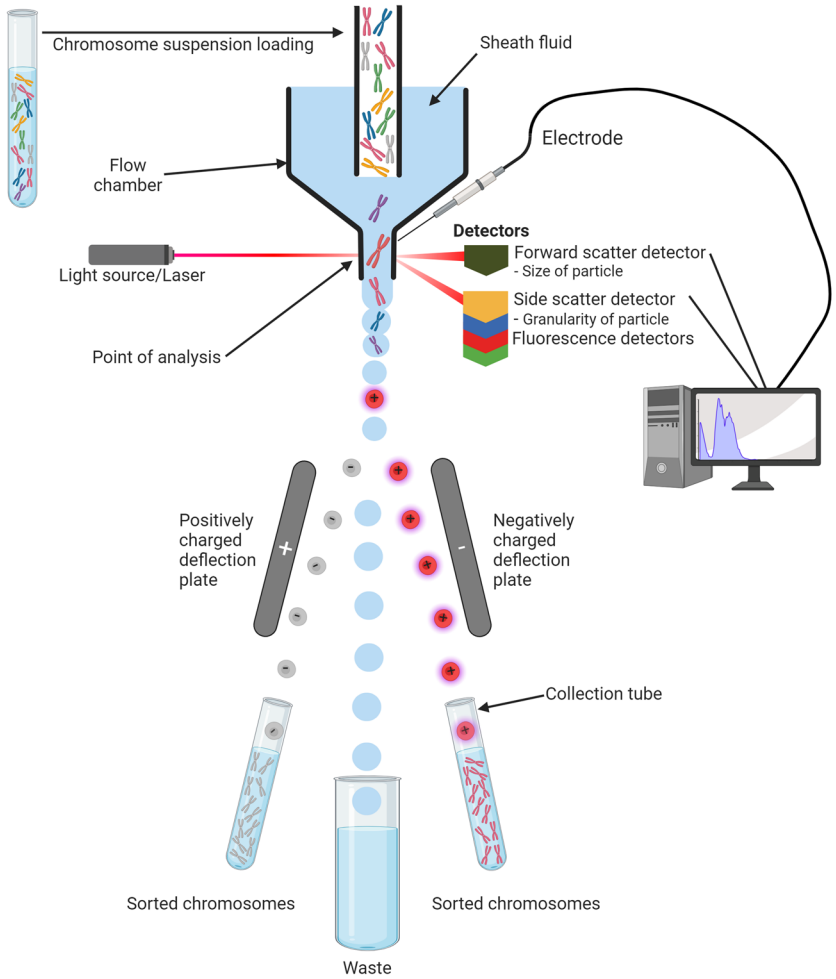

Fig. 1 A simplified scheme of a flow cytometer and sorter. Chromosomes in suspension are arranged into a single file and interact with a laser beam. Scattered light is captured in the direction of the beam and at a right angle to it. Moreover, fluorescence of stained chromosomes is also captured. Pulses from both scattered light and fluorescence are transmitted to detectors and converted into electrical signals. These signals are then digitized and processed by a computer. To facilitate chromosome sorting, the liquid stream is broken into small droplets. The droplets carrying the chromosomes of interest are electrically charged and deflected towards collection vessels by a passage through an electrostatic field created by two deflection plates

This approach offered numerous advantages. Seeds are available in a majority of species, and by placing germinating seedlings on a hydroponic system, roots can be treated with cell cycle synchronizing agents dissolved in a nutrient solution. Cycling meristem root tip cells are easy to synchronize and, unlike in vitro cultured cells, are karyologically stable. Veuskens et al. [110] and Neumann et al. [76] showed that genetically transformed "hairy" root cultures may be used when particular cytogenetic stocks cannot be maintained by seed propagation. In fact, roots suitable for chromosome isolation may be induced from vegetative parts of plants, such as the setts in sugarcane [71], or bulbs in onion and garlic (unpublished).

With the aim to develop a generally applicable protocol for chromosome release from synchronized root tips, Doležel et al. [16] avoided enzymatic treatments and optimized a procedure based on mechanical homogenization. In order to make chromosomes resistant to mechanical shearing forces, the roots were fixed mildly in formaldehyde prior to homogenization. The mechanical stability means that chromosomes are stable during the analysis and also during sorting, and flow-sorted chromosomes can be easily identified. Importantly, the formaldehyde fixation increases the yield of isolated chromosomes [16]. Much later the formaldehyde fixation turned out to be an advantage when flow-sorted chromosomes were used for chromosome conformation capture analysis, which requires chromatin cross-linking [50]. In the original protocol [16], root tips were homogenized by chopping using a sharp scalpel. This was laborious and not practical for small roots. Thus, Gualberti et al. [30] modified the protocol so that roots are homogenized using a mechanical homogenizer, making the method suitable for small roots, such as those in cereals (Fig. 2).

In order to preserve chromosomes and their DNA intact, chromosomes should be released into a buffer with appropriate composition [20]. The protocol of Doležel et al. [16] used LB01 buffer developed for the isolation of plant cell nuclei [15]. This buffer is suitable for a majority of downstream applications of sorted chromosomes. However, it had to be modified to prepare high molecular weight DNA and the modified version of LB01 is referred to as HKS (originally IB) [96]. Chromosomes used for proteomic analyses were isolated in LB01-P which contained protease inhibitor phenylmethylsulfonyl fluoride [83].

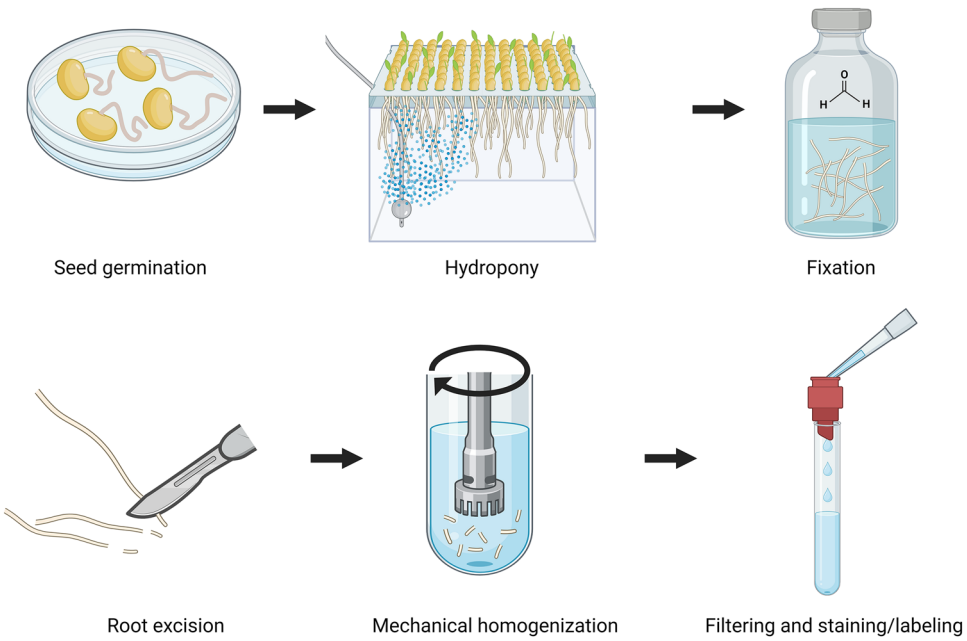

Fig. 2 Preparation of chromosome suspension for flow cytometry: Seeds are initiated into germination on dampened tissue paper within a Petri dish until the roots extend approximately 2 cm in length. The young seedlings are placed onto a plastic cover lid and positioned onto a tray, ensuring that the roots are fully immersed in a well-aerated nutrient solution at 25 °C. Cycling meristem tip cells are accumulated at G_1/S interphase by treatment with hydroxyurea, the cells then resume DNA synthesis and traverse S and G_2 phases of cell cycle. Once mitosis is reached, the cells are accumulated at metaphase by treatment with a mitotic spindle inhibitor. Immediately following this, the roots are fixed by formaldehyde. Meristem root tips are then excised and mechanically homogenized to release chromosomes into a solution. Prior to flow cytometric analysis, crude chromosome suspension is strained through a nylon mesh and chromosomal DNA is stained by suitable fluorochrome. Repeated DNA sequence may be fluorescently labelled at this stage

Flow karyotyping

Discrimination of particular chromosomes

In some applications it is not necessary to discriminate chromosomes from each other and it is enough to resolve the population of mitotic chromosomes from other particles in the sample. However, in a number of uses the aim is to discriminate a particular chromosome, or a group of a few chromosomes. Given that only relative fluorescence and light scattering parameters are measured, discrimination of particular chromosomes has been a challenge in a majority of plants. Two principal and compatible strategies were developed to aid in chromosome discrimination. The first relies on optical parameters and the second on the karyotype itself and its modifications.

Considering the information derived from the optical signals, if the chromosomes in suspension are stained by a DNA fluorochrome, the fluorescence pulse area will reflect relative size of chromosomes if DNA intercalator has been used, or AT/GC content if dyes binding preferentially to AT or GC-rich DNA regions are used. If samples are prepared following the protocol of Doležel et al. [16] from formaldehyde-fixed tissues, staining chromosomes by DAPI was found superior over staining with DNA intercalators and GC-binding fluorochromes [18]. While the width of the fluorescence pulse indicates the relative chromosome length, the pulse height indicates the highest fluorescence signal along the chromosome. Light scattering signals are less informative, but are useful to discriminate chromosome population from debris, which is not fluorescent and not detected by fluorescence analysis and hence may contaminate flow sorted chromosomes.

The results of fluorescence and light scattering analysis are typically displayed as monoparametric histograms called flow karyotypes. Ideally, a flow karyotype comprises well separated peaks, each representing different chromosomes in the karyotype. However, this is a rare case in plants and usually one or more peaks represent more than one chromosome. To obtain high resolution flow karyotypes, light scatter and fluorescence pulses are displayed as a biparametric dot-plot and a gating region is set on it to exclude debris, nuclei, and large clumps. This gate is applied to other histograms and dot-plots (Fig. 3).

The ability to perform high resolution analysis and achieve chromosome discrimination depends on several parameters with the sample quality being one of the most important. Chromosome suspensions should have high concentration of intact chromosomes (10^5–10^6/ml) and small proportions of single chromatids, cells and chromosome debris. It is also critical that the flow cytometer laser beams are perfectly aligned and stable so that optical parameters are measured at the highest resolution. If, however, two or more chromosomes in the sample have the same relative fluorescence, they cannot be resolved by the analysis of a single fluorescence parameter. The number of chromosomes which can be discriminated after monovariate flow karyotyping in plants varies between species (Table 1, [17]). While only one out of the 21 chromosome pairs of bread wheat can be discriminated from a wild-type karyotype [114] five out of eight chromosome pairs can be resolved in chickpea [112].

Contrary to the great improvement of chromosome resolution in human and animals, simultaneous staining with AT- and GC-binding fluorochromes was not found useful in plants [53, 56, 61]. On the other hand, a dramatic improvement in the ability to resolve chromosomes was achieved after a protocol for fluorescence in situ

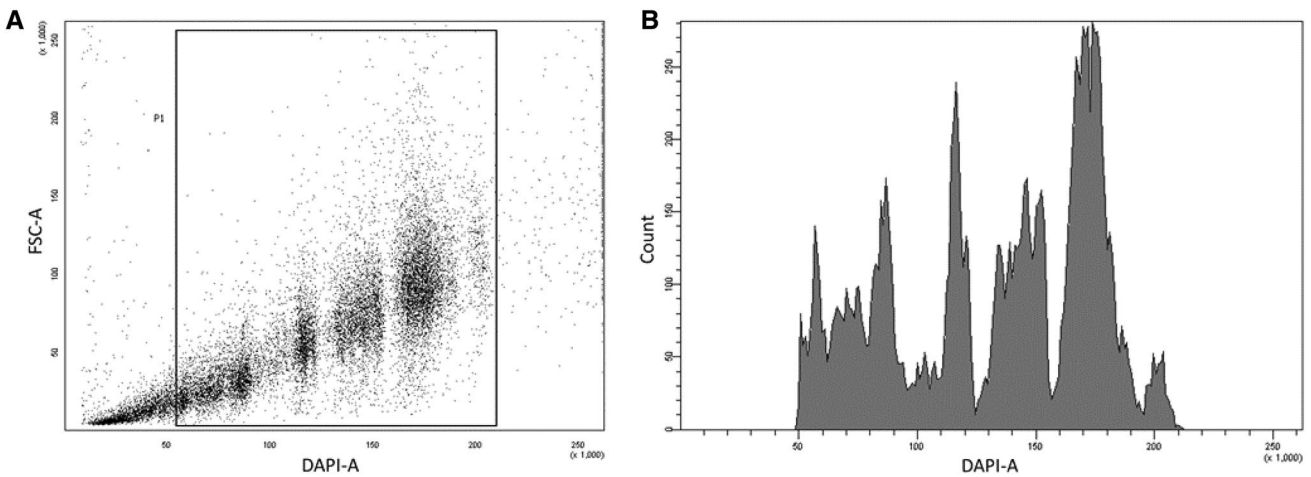

Fig. 3 Chromosome analysis in bread wheat (*T. aestivum*, $2n = 6x = 42$) cv. Chinese Spring. **A** First, a primary gate (P1) is set on dot-plot DAPI fluorescence pulse area (DAPI-A) versus forward scatter pulse area (FSC-A) to select only intact chromosomes and chromatids. **B** Signals originating from the particles in the P1 gate are displayed as monoparametric flow karyotype DAPI-A on which the three large composite peaks, each representing a group of chromosomes, and a small peak of chromosome 3B are recognized [19]

Table 1 List of plant species from which flow cytometric analysis of mitotic chromosomes has been reported

Species		Chromosome number (n)[a]	Number of discriminated chromosomes and/or arms		References
Latin name	Common name		Standard karyotype[b]	Cytogenetic stocks[c]	
Aegilops biuncialis	Goatgrass	14	9	6	Molnár et al. [72], Said et al. [88]
Aegilops comosa	Goatgrass	7	1		Molnár et al. [72]
Aegilops cylindrica	Goatgrass	14	4		Molnár et al. [74]
Aegilops geniculata	Goatgrass	14	14	14	Molnár et al. [72], Said et al. [88]
Aegilops markgrafii	Goatgrass	7	4		Molnár et al. [74]
Aegilops tauschii	Goatgrass	7	1		Molnár et al. [73]
Aegilops triuncialis	Goatgrass	14	2		Molnár et al. [74]
Aegilops umbellulata	Goatgrass	7	4		Molnár et al. [72]
Agropyron cristatum	Crested wheatgrass	7, 14	0	12	Said et al. [89, 90]
Asparagus officinalis	Sparrow grass	10	5		Moreno et al. [75]
Cicer arietinum	Chickpea	8	5		Vláčilová et al. [112], Zatloukalová et al. [124]
Dasypyrum villosum (syn. *Haynaldia villosa*)	Mosquito grass	7	1		Grosso et al. [29], Wang et al. [115]
Festuca pratensis	Meadow fescue	7	1		Kopecký et al. [42, 43]
Haplopappus gracilis		2	2		de Laat and Blaas [51], de Laat and Schel [14]
Hordeum vulgare	Barley	7	1 (2)	7	Lysák et al. [63], Lee et al. [53], Suchánková et al. [102]
Lycopersicon pennellii	Tomato	12	2		Arumuganathan et al. [4, 5]
Petunia hybrida		7	1		Conia et al. [11]
Picea abies	Norway spruce	12	3		Überall et al. [108]
Pisum sativum	Pea	7	2	4	Gualberti et al. [30], Neumann et al. [76, 77]
Secale cereale	Rye	7	1	7	Kubaláková et al. [48]
Silene latifolia [syn. *Melandrium album*)	White campion	12	2		Veuskens et al. [110], Kejnovský et al. [39], Kralova et al. [45], Vrána et al. [113]
Triticum aestivum	Bread wheat	21	1 (2)	21	Wang et al. [116], Schwarzacher et al. [94], Lee et al. [56], Gill et al. [24], Vrána et al. [114], Kubaláková et al. [49], Giorgi et al. [25]
Triticum dicoccoides	Wild emmer	14	0	14	Akpinar et al. [3], Vrána et al. [113]
Triticum durum	Durum wheat	14	1	14	Kubaláková et al. [47], Giorgi et al. [25], Vrána et al. [113]
Vicia faba	Field bean	6	1	6	Doležel et al. [16]; Lucretti et al. [62], Doležel and Lucretti [18], Lucretti and Doležel [61], Kovářová et al. [44]
Vicia sativa	Common vetch	6	2		Kovářová et al. [44]
Zea mays	Maize	10	2 (3)	10	Lee et al. [54, 55], Li et al. [58, 59], Vrána et al. [113]

[a]Number of chromosomes in a haploid set

[b]Number of chromosomes that could be discriminated unambiguously. The numbers in parentheses indicate the number of chromosomes that could be discriminated in some lines due to chromosome polymorphism

[c]Number of individual chromosomes and/or arms discriminated in different cytogenetic stocks (translocation, deletion, telosome or chromosome addition lines). Note that in some species this option has not been verified

hybridization in suspension (FISHIS) was developed [25]. The ability to fluorescently label particular repetitive DNA sequences such as microsatellites provided a signal independent of chromosome DNA or base-pair content. For example, while monovariate flow karyotyping in bread wheat resolved only one out of the 21 chromosomes, bivariate analysis DAPI versus FITC of GAA microsatellite fluorescence resolved the whole genome set of 21 chromosomes of bread wheat [88].

As shown already by Doležel and Lucretti [18] altered chromosome length and structure resulting in changed chromosome length helps to increase the number of resolvable chromosomes. Successful examples include *A. cristatum* ditelosomic lines [90, 125] and rye ditelosomic lines, wheat-rye and wheat-wheat chromosome translocations [49] as well as wheat-*D. villosum* translocation lines [60].

Chromosomes with altered length and structure due to translocation may not only be resolvable, but the change in chromosome size in some cases may reveal some wild-type chromosomes, which are otherwise not resolved on flow karyotype [76]. An obvious disadvantage for downstream molecular analysis of sorted translocation chromosomes is that they do not represent wild-type chromosomes. On the other hand, sorting the translocations allowed mapping of DNA sequences to subchromosomal regions by PCR [63] and to characterize chromosome breakpoints by sequencing [46]. In analogy, alien chromosome-and chromosome arm addition lines have one or more chromosomes introduced from other species and if they differ in optical parameters, they can be discriminated. The examples include wheat-rye [49], wheat-*A. cristatum* [90, 125], wheat-*Ae. biuncialis* and wheat-*Ae. geniculata* [88] translocation and disomic and ditelosomic alien introgression lines.

Applications of flow karyotyping

Due to the limited information content of fluorescence and light scattering signals, flow karyotyping cannot compete with microscopic chromosome analysis. Yet, a number of reports describe identification of structural and numerical chromosome changes by flow karyotyping. These, include identification of trisomy of chromosome 6 in barley [53] and determining the frequency of alien chromosomes in wheat–rye chromosome addition lines [48]. Translocation chromosomes were identified in field bean, garden pea, barley, and wheat [18, 48, 49, 63, 76, 114], and chromosome deletions were identified in wheat [24, 47, 49]. Intraspecific variation in chromosome structure (chromosome polymorphism) was observed in barley, maize, rye, and wheat [48, 49, 53, 55, 114].

Flow karyotyping identified alien chromosomes in oat–maize and wheat–rye chromosome addition lines [48, 59] and alien chromosome arms were identified in wheat–rye and wheat–barley telosomic addition lines [97, 102]. Flow karyotyping was also suitable to detect accessory B chromosomes in rye, and sex chromosomes in white campion [48, 110]. Figure 4 shows examples of flow karyotyping in bread wheat, wheat-barley 7HS ditelosomic addition line and wheat chromosome translocation line. Despite the successful examples of detecting chromosome translocations, deletions and alien additions the most important application of flow karyotyping has been the identification of chromosomes that are to be sorted.

Chromosome sorting

Sorting of required chromosomes

Depending on differences in optical properties of chromosomes in a karyotype and the way gate windows are set, flow cytometer may purify a chromosome, a group of chromosomes, or a complete chromosome complement. The decision depends on downstream application of sorted chromosomes and on the ability to resolve required chromosomes. For the latter, the resolution of flow karyotype is critical and the contamination in the sorted fraction by other particles depends on the way how well the sorted chromosomes are discriminated from other chromosomes. In order to characterize the sorted chromosome population, chromosomes are sorted onto a microscope slide, dried and identified after fluorescence in situ hybridization (FISH) with probes that give chromosome-specific patterns (Fig. 5). Although laborious, this approach permits identification of all chromosomes in the sorted fraction and determination of the frequency of other chromosomes and chromosome arms that contaminate the sorted fraction.

If, however, downstream analysis is to be performed with a particular chromosome in the karyotype and the chromosome cannot be resolved by any of the approaches discussed in section "Discrimination of particular chromosomes", the option is to sort single copies of anonymous chromosomes and amplify their DNA individually [9]. Amplified DNA samples are then assigned to particular chromosomes using PCR with chromosome-specific primers. The samples originating from the same chromosome are then pooled to improve the chromosome sequence coverage before further analysis. Alternatively, amplified DNA samples are sequenced and reads originating from the chromosomes of interest are merged [31, 46].

Applications of flow-sorted chromosomes

The flow-sorted chromosomes found attractive applications in genome mapping, gene cloning and genome sequencing. The main advantage being the fact that sorting dissects genome to small and defined parts, allowing for targeted and hence simplified and more economic analyses as compared to whole genome approaches.

However, even non-targeted, i.e., whole genome analyses, may greatly benefit from the purification of complete chromosome complement. As flow-sorted chromosomes are intact and DNA prepared from them is of high molecular weight DNA [96], purified chromosomes are a superior source of DNA for the construction of large-insert BAC (Bacterial Artificial Chromosome) libraries [86, 87], optical mapping [99] as well as for proteomic analyses, as the chromosomal proteins are also well maintained [82, 83]. Other applications, which do not rely on discriminating particular chromosomes and for which complete chromosome complement is sorted, comprise mapping DNA sequences using FISH [37, 48], including FISH with longitudinally extended chromosomes to improve spatial resolution [22, 109]. The advantage of using flow-sorted chromosomes for molecular cytogenetics is that a large number of chromosomes are spread over a small area of microscope slide and the preparations are free of cellular debris and cytoplasm. Recently, purified mitotic chromosomes were found useful to analyze chromosome surface (perichromosomal layer) using advanced environmental scanning electron microscopy [Neděla et al., in preparation].

The uses of flow sorted chromosomes for genome complexity reduction and targeted analysis keep on expanding hand in hand with the progress in the methods of molecular biology and genomics (Fig. 6).

The construction of short insert (10^2–10^3 bp) DNA libraries, such as the library enriched for chromosome 4A of bread wheat by Wang et al. [116] and a library for chromosome 2 of wild tomato (*Lycopersicon pennellii*) by Arumuganathan et al. [4] were among the early applications of flow-sorted chromosomes. The first complete set of chromosome-specific DNA libraries covering a whole plant genome was constructed by Macas et al. [64] in field bean (*Vicia faba*). Due to short inserts, the libraries were mainly used to develop DNA markers, such as microsatellites [40, 64, 84]. After a method was optimized for the construction of chromosome (arm)-specific BAC libraries

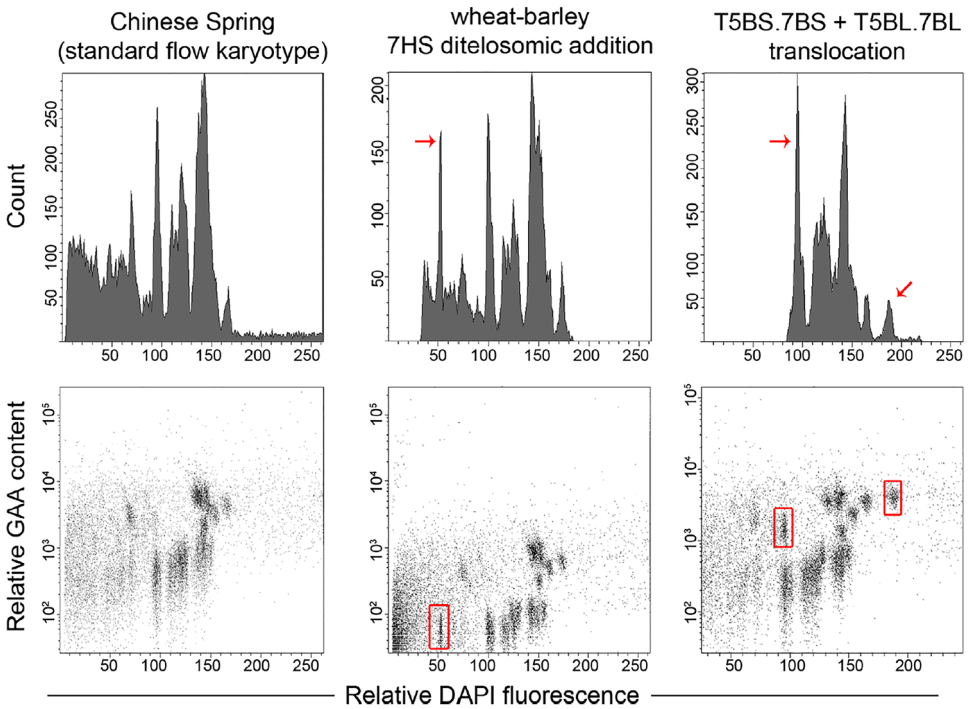

Fig. 4 Chromosome discrimination in bread wheat cv. Chinese Spring with a wild-type karyotype (left column), wheat-barley 7HS ditelosomic addition line (middle column) and wheat chromosome translocation line T5BS•7BS + T5BL•7BL (right column). The upper row shows monovariate flow karyotypes obtained after the analysis of DAPI fluorescence pulse area (DAPI-A) and red arrows point to peaks representing chromosomes, which can be easily discriminated. The lower row shows bivariate flow karyotypes DAPI-A (*x*-axis) versus FITC-A (*y*-axis) and red rectangles define the populations of chromosomes represented by peaks in monovariate flow karyotypes (upper row). Note that the small translocation chromosome T5BS•7BS is included in the composite peak of wild-type chromosomes 1D, 4D, and 6D on a monovariate flow karyotype, while it is clearly discriminated in the bivariate flow karyotype [19]

[86], in addition to the construction of physical maps [81] and map-based sequencing [10] the libraries were used to develop DNA markers, such as the insertion site-based polymorphism (ISBP) markers from rye chromosome 1R [7]. Another approach for targeted development of DNA markers was pioneered by Wenzl et al. [119] who developed DArT markers [36] from chromosome 3B and chromosome short arm 1B of bread wheat.

The advent of the next generation sequencing, which opened new horizons for plant genomics, provided new opportunities for the application of flow-sorted chromosomes, including high-throughput development of DNA markers. As the short-read technologies did not require high molecular weight DNA, microgram amounts of DNA obtained from only tens of thousands of chromosomes after multiple displacement amplification of chromosomal DNA [98] were suitable. Successful uses included *in silico* identification of microsatellite loci from the long arm of bread wheat chromosome 7D [78] and identification of almost 10^7 transposable element junctions from barley (*Hordeum vulgare*) chromosome 1H and from twelve arms of the remaining six barley chromosomes [70]. Chromosome-derived sequences were also used to develop intron length polymorphism markers [115] and SNP markers [95, 100] to trace alien chromatin in the background of bread wheat and clone resistance genes [79, 106].

Importantly, shotgun sequencing of flow-sorted chromosomes was useful to assemble draft chromosome sequences in some crops and their wild relatives. After a pioneering work of Mayer et al. [69] who characterized DNA repeats, identified genic sequences and constructed a virtual gene order map of barley chromosome 1H, this approach was used to create draft sequences from the B chromosome of rye [66], chromosome group 1 [120], chromosome 4A [32] and chromosome 5A [111] of bread wheat. In wild relatives of the crop, draft assemblies were created from chromosome 5D of *Ae. tauschii* [3], chromosome $5M^g$ of *Ae. geniculata* [105] and the short arm of chromosome 4V of *H. villosa* [121]. The availability of chromosome sequences made it possible to characterize molecular organization, create virtual gene orders ("genome zippers") and describe evolutionary structural changes.

More ambitious projects employed chromosome sequencing to create draft assemblies of the whole genomes. The choice of the chromosome-by-chromosome strategy streamlined the assembly of large and complex genomes characterized by sequence redundancy. In the first attempt of this kind, Mayer et al. [68] developed the first draft sequence of the barley genome. As barley chromosomes 2H–7H cannot be discriminated from each other, the team flow-sorted chromosome arms from bread wheat-barley telosome addition lines. Sequencing the arms allowed location of centromeric regions and characterize their DNA sequences. Chromosome 1H was purified from barley genotype with wild-type karyotype. A similar strategy was used to create the first draft genome sequence of rye, when chromosome 1R was flow-sorted directly from a rye genotype with standard karyotype and chromosomes 2R–7R were purified from wheat-rye disomic chromosome addition lines [67]. The third and by far the most ambitious study was the production of the first draft genome of hexaploid bread wheat [35]. As before the discovery of FISHIS [25] only chromosome 3B could be discriminated from other chromosomes, the project relied on sorting chromosome arms from bread wheat telosomic lines.

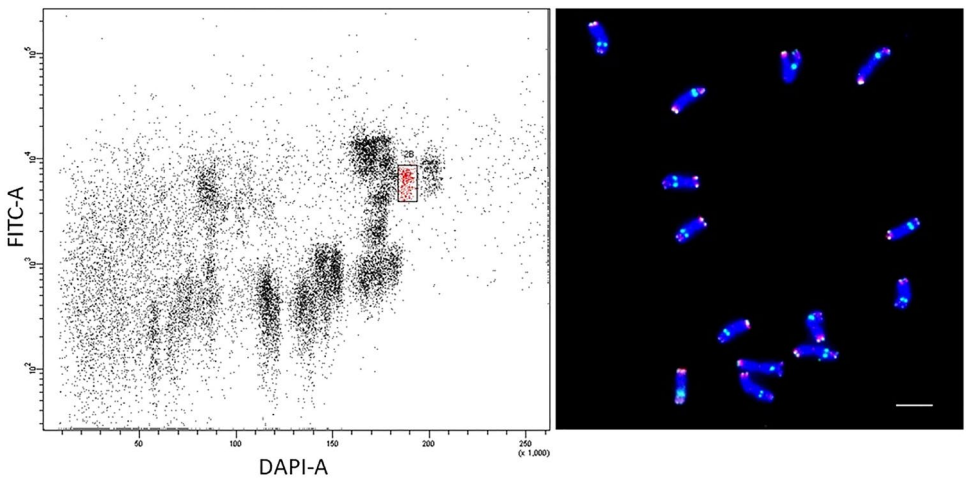

Fig. 5 Flow sorting of chromosomes 2B from bread wheat cv. Chinese Spring. Left: bivariate flow karyotype DAPI-A vs. FITC-A; right: Images of flow-sorted chromosomes 2B after FISH. Prior to flow cytometric analysis and sorting, chromosomes in suspension were fluorescently labeled by FISHIS using oligonucleotide probe 5′-FITC-$(GAA)_7$-FITC-3 and counterstained with DAPI (4′,6-diamidino 2-phenylindole). Chromosome content and purity in flow-sorted fractions were determined by FISH on chromosomes sorted onto microscope slides using probes for pSc119.2 and AFA family repeat. Bar = 10 µm

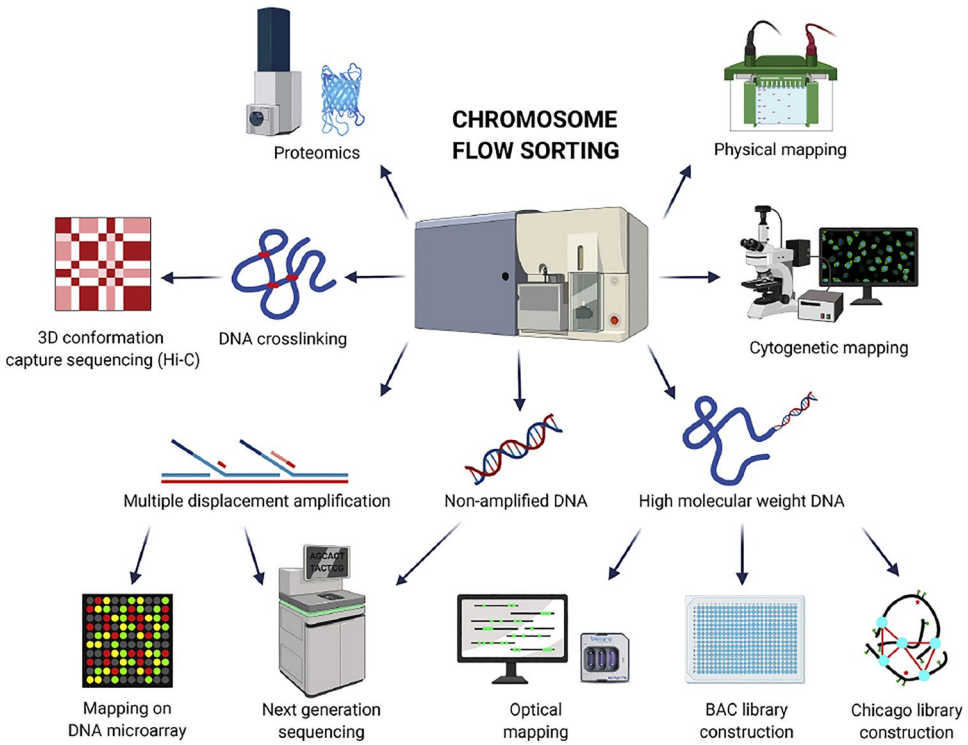

Fig. 6 Overview of methods using flow-sorted chromosomes, their DNA, or proteins [126].

While the draft genomes of barley and rye were developed using the Roche 454 sequencing technology [67, 68], wheat chromosomes were sequenced using Illumina technology. This technology was used to sequence flow-sorted chromosomes in all projects that followed these pioneering works. The quality of chromosome assemblies was later improved after the Illumina company introduced improved protocols for preparation of sequencing libraries, which required only nanograms of DNA. This made it possible to avoid the chromosome amplification step, which was not completely representative [21].

An interesting application of chromosome sequencing has been the validation of whole genome assemblies, which may suffer from misplaced or chimeric contigs and scaffolds. Thus, Ruperao et al. [85] validated genome assemblies of chickpea (*Cicer arietinum*) kabuli and desi varieties by mapping chromosome sequence reads to genome assemblies of both varieties. This work revealed misassembled regions and facilitated improvement of the assemblies. Later, Kreplak et al. [46] used chromosome-derived sequences during the development of the reference genome of pea (*Pisum sativum*). The study was primarily based on whole genome sequencing and mapping chromosome-derived reads to the genome assembly, served to identify scaffolds that contained contigs from different chromosomes. Kreplak et al. [46] also sequenced chromosomes purified from pea wild relatives *P. fulvum*, *P. sativum* subsp. *elatius* and *P. sativum* subsp. *humile* to identify evolutionary chromosome translocations. As a majority of chromosomes in *Pisum* species cannot be discriminated and sorted, single copies of chromosomes were sorted and their DNA amplified prior to sequencing.

A decrease in DNA sequencing costs, advent of long read sequencing and improvement in genome assemblers made chromosome sorting not necessary for whole genome sequencing, perhaps with the exception of very complex genomes such as that of sugarcane [Healey et al., in preparation]. Thus, a number of recent applications deal with gene mapping and cloning. The examples include mapping powdery mildew resistance gene *QPm-tut-4A* introduced to bread wheat from *T. militinae* [2], mapping leaf rust and stripe rust resistance genes introduced from *Ae. umbellulata* to bread wheat and identification of candidate *Lr76* and *Yr70* genes by Bansal et al. [6], mapping and isolating *SuSr-D1* gene suppressing stem rust resistance in bread wheat [33] and mapping Russian wheat aphid resistance gene *Dn2401* in bread wheat [107]. The complete and accurate sequence of the *Dn2401* region facilitated the identification of new markers and precise annotation of the interval, revealing six high-confidence genes, where the *Epoxide hydrolase 2* was identified as the most likely *Dn2401* candidate gene for aphid resistance [107].

To support gene cloning, Thind et al. [104] developed an approach called "targeted chromosome-based cloning via long-range assembly" (TACCA), which relies on the production of high-quality *de novo* assembly from flow-sorted chromosome. In fact, it is an alternative to the standard whole genome map-based gene cloning and the extra work with chromosome sorting is compensated

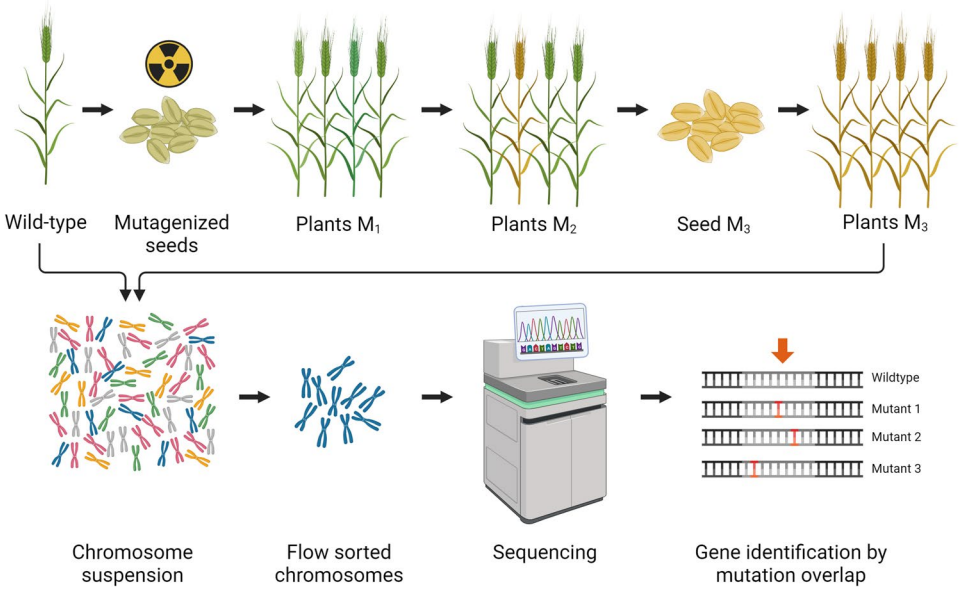

Fig. 7 MutChromSeq strategy for rapid gene cloning [91]. Seeds of wild-type plant are subjected to mutagenesis, followed by the identification of mutant plants in the M2 generation and assessment of their traits in the M3 generation. Next, the chromosome bearing the gene is flow sorted from the wild-type plant and from several mutants and chromosomal DNA is sequenced. Comparative sequence analysis identifies the candidate gene

by greatly reduced sample complexity. In their pioneering work, Thind et al. [104] used TACCA to clone leaf rust resistance gene *Lr22a* introgressed to bread wheat from *Ae. tauschii*. They were followed by Xing et al. [122] who used TACCA to clone powdery mildew resistance gene *Pm21*, which was introduced to bread wheat from *Haynaldia villosa* and Holden et al. [34] who discovered that *Rps8* locus on chromosome 4H of barley comprises genetic module including *Pur1* and *Exo70FX12*, each of which is necessary and together sufficient for *Rps8*-mediated resistance to wheat stripe rust.

To date, the most successful approach to clone genes using flow-sorted chromosomes has been the "mutant chromosome sequencing" (MutChromSeq), which involves comparing sequences from wild-type parental chromosome to chromosomes from a few independently derived mutants to identify causative mutations in a candidate gene (Fig. 7). Developed by Sánchez-Martín et al. [91], MutChromSeq does not require high-resolution genetic mapping and does not exclude any DNA sequence from being targeted. It permits rapid cloning of genes and regulatory sequences with a strong phenotype. The authors verified MutChromSeq by re-cloning barley *Eceriferum-q* gene and cloned wheat powdery mildew resistance gene *Pm2*. A series of MutChromSeq applications that followed includes cloning of semi-dwarfism gene in bread wheat [23], leaf rust resistance gene *Rph1* from barley chromosome 2H [21], *SuSr-D1* gene that suppresses resistance to stem rust from chromosome 7D of bread wheat, *LYS3* gene controlling lysine content from chromosome 5H of barley [80], bread wheat *Pm4* race-specific resistance gene to powdery mildew from chromosome 2A [92], race-specific leaf rust resistance gene *Lr14a* from bread wheat chromosome 7B [41], an alternative dwarfing gene *Rht13* from bread wheat chromosome 7B, which encodes an autoactive *NB-LRR* gene rather than a component of gibberellin signaling or metabolism [8], leaf rust resistance genes *Lr9* and *Lr58* which were introgressed into bread wheat from *Ae. umbellulata* and *Ae. triuncialis*, respectively [117] and stem rust resistance gene *Sr43*, which was introgressed into bread wheat from wild relative *Thinopyrum elongatum* [123].

Conclusions

Flow cytogenetics stands as a very useful and versatile tool with a spectrum of applications. From the classification of chromosomes to the isolation of specific ones, it has played a pivotal role in advancing plant genomics and molecular biology. Particularly noteworthy is its role in expediting the sequencing of complex genomes of important agricultural crops, as well as in rapid identification of genes that underline traits affecting plant growth and resistance to pests and diseases as well as the topography of condensed mitotic chromosomes. One notable bottleneck has been the discrimination of individual chromosomes. To address this, the incorporation of spectral cytometry shows promise in augmenting the array of probes utilized for FISHIS, thereby enhancing the power of chromosome discrimination. Furthermore, the integration of image flow cytometry could further refine this process. A wider application of flow-sorted chromosomes could be spurred by the development of protocols for preparation of high molecular weight DNA suitable for long-read DNA sequencing. As we forge ahead, the continued refinement of flow cytogenetics holds immense potential for unraveling the intricacies of genetics and enhancing our understanding of fundamental biological processes.

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Declarations

Conflict of interest The authors declare no conflict of interest in this study.

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